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Inhibition of opioid release in the rat spinal cord by α_{2C} adrenergic receptors

Wenling Chen [a,b], Bingbing Song [a,b,1], Juan Carlos G. Marvizón [a,b,*]

[a] Center for the Neurobiology of Stress and CURE: Digestive Diseases Research Center, Division of Digestive Diseases, Department of Medicine, David Geffen School of Medicine at UCLA, Los Angeles, CA 90095, USA
[b] Veteran Affairs Greater Los Angeles Healthcare System, Los Angeles, CA 90073, USA

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Abstract

Neurotransmitter receptors that control the release of opioid peptides in the spinal cord may play an important role in pain modulation. Norepinephrine, released by a descending pathway originating in the brainstem, is a powerful inducer of analgesia in the spinal cord. Adrenergic α_{2C} receptors are present in opioid-containing terminals in the dorsal horn, where they could modulate opioid release. The goal of this study was to investigate this possibility. Opioid release was evoked from rat spinal cord slices by incubating them with the sodium channel opener veratridine in the presence of peptidase inhibitors (actinonin, captopril and thiorphan), and was measured in situ through the internalization of μ-opioid receptors in dorsal horn neurons. Veratridine produced internalization in 70% of these neurons. The α_2 receptor agonists clonidine, guanfacine, medetomidine and UK-14304 inhibited the evoked μ-opioid receptor internalization with IC_{50}s of 1.7 μM, 248 nM, 0.3 nM and 22 nM, respectively. However, inhibition by medetomidine was only partial, and inhibition by UK-14304 reversed itself at concentrations higher than 50 nM. None of these agonists inhibited μ-opioid receptor internalization produced by endomorphin-2, showing that they inhibited opioid release and not the internalization itself. The inhibitions produced by clonidine, guanfacine or UK-14304 were completely reversed by the selective α_{2C} antagonist JP-1203. In contrast, inhibition by guanfacine was not prevented by the α_{2A} antagonist BRL-44408. These results show that α_{2C} receptors inhibit the release of opioids in the dorsal horn. This action may serve to shut down the opioid system when the adrenergic system is active.

Keywords: Clonidine; Dorsal horn; Dynorphin; Enkephalin; Guanfacine; Internalization; JP-1302; Medetomidine; mu-Opioid receptor; Norepinephrine; Opioid; UK-14304

1. Introduction

Norepinephrine and opioid peptides (henceforth, "opioids") are among the most powerful inducers of analgesia in the spinal cord. The analgesic effect of opioids (Chen and Pan, 2006; Russell et al., 1987) is mediated by μ-opioid receptors (MORs) and δ-opioid receptors (Budai and Fields, 1998; Chen et al., 2007b; Takemori and Portoghese, 1993), while that of norepinephrine is largely mediated by adrenergic α_2 receptors (Pertovaara, 2006; Yaksh, 1985). Physiologically, norepinephrine-induced analgesia is driven by a descending pain inhibitory pathway originating in three adrenergic nuclei: A5, A7 and nucleus coeruleus (A6) (Pertovaara, 2006). Spinal cord opioids derive from the pre-proenkephalin and pre-prodynorphin genes, which are expressed in different interneurons (Cruz and Basbaum, 1985). At present, it is not clear to what extent spinal opioid release is driven by a brainstem descending pathway (Jensen and Yaksh, 1984; Morgan et al., 1991; Zorman et al., 1982) or by local dorsal horn circuits (Cesselin et al., 1989; Le Bars et al., 1987a,b; Song and Marvizon, 2003b). Synergism between the analgesic effects of opioid and α_2 adrenergic receptors (Bohn et al., 2000; Stone et al., 1997; Wei et al., 1996)

* Corresponding author. Veteran Affairs Greater Los Angeles Healthcare System, Los Angeles, CA 90073, USA. Tel.: +1 310 478 3711x41850; fax: +1 310 312 9289.
E-mail address: marvizon@ucla.edu (J.C.G. Marvizón).
[1] Present address: UCLA Neurobiology, Box 951763, 504 NRB, Los Angeles, CA 90095, USA.

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suggests that there are separate noradrenergic and opioidergic pain inhibitory pathways. However, the mechanisms by which these two pathways interact with each other remain unclear.

Of the three subtypes of α_2 receptors, α_{2A} receptors are present in the primary afferent terminals and α_{2C} receptors are located in the presynaptic terminals of interneurons (Marvizon et al., 2007; Olave and Maxwell, 2002, 2003a,b, 2004; Stone et al., 1998), whereas expression of α_{2B} receptors in the dorsal horn is quite limited (Shi et al., 1999). Of the presynaptic terminals with α_{2C} receptors, 84% are excitatory, since they contain the vesicular glutamate transporter 2 (VGLUT2), and 11% are inhibitory, based on the presence of glutamic acid decarboxylase (Olave and Maxwell, 2003a). Noradrenergic analgesia is thought to be mediated primarily by α_{2A} receptors, which inhibit glutamate release from primary afferent terminals (Li and Eisenach, 2001). Additionally, α_2 receptors hyperpolarize dorsal horn neurons (North and Yoshimura, 1984). Noradrenergic analgesia may also involve α_{2C} receptors (Fairbanks et al., 2002), which could block excitatory input onto projection neurons (Olave and Maxwell, 2003a,b, 2004).

The fact that presynaptic terminals that have α_{2C} receptors also contain opioids (Marvizon et al., 2007; Olave and Maxwell, 2002; Stone et al., 1998) raises the possibility that α_{2C} receptors control opioid release. They probably inhibit opioid release, because α_2 receptors couple to α_i G-proteins and inactivate Ca^{2+} channels (Summers and McMartin, 1993). We investigated this possibility by studying the effect of α_2 agonists and antagonists on opioid release evoked in rat spinal cord slices by veratridine (Przewlocka et al., 1990; Song and Marvizon, 2003a; Song and Marvizon, 2005; Uzumaki et al., 1984). Opioid release was measured in situ by the internalization of MORs, an approach validated in the spinal cord (Chen et al., 2007b; Song and Marvizon, 2003a,b; Song and Marvizon, 2005; Trafton et al., 2000) and the brain (Eckersell et al., 1998; Mills et al., 2004; Sinchak and Micevych, 2001). MOR internalization correlates well with opioid-induced analgesia and other measures of MOR activation (Chen et al., 2007b; Marvizon et al., 1999; Trafton et al., 2000). Parts of this study have been published as an abstract (Chen et al., 2007a).

2. Methods

Animal procedures were approved by the Institutional Animal Care and Use Committee of the Veteran Affairs Greater Los Angeles Healthcare System, and conform to NIH guidelines. Efforts were made to minimize the number of animals and their suffering.

2.1. Spinal cord slices

Coronal spinal cord slices were prepared as previously described (Marvizon et al., 1999; Song and Marvizon, 2003a,b; Song and Marvizon, 2005). Media used for the slices were: aCSF, containing (in mM): 124 NaCl, 1.9 KCl, 26 $NaHCO_3$, 1.2 KH_2PO_4, 1.3 $MgSO_4$, 2.4 $CaCl_2$ and 10 glucose; K^+-aCSF, containing 5 mM of KCl, and sucrose-aCSF, the same as K^+-aCSF except that NaCl was iso-osmotically replaced with sucrose (215 mM). The spinal cord was extracted from 3–4 week old male Sprague–Dawley (Harlan, Indianapolis, IN) rats under isoflurane anesthesia. Coronal slices (400 μm) were cut with a Vibratome (Technical Products International, St. Louis, MO) in ice-cold sucrose-aCSF. Up to six slices from each animal were cut sequentially in the L1-L4 region.

2.2. Slice stimulation

To evoke opioid release, slices were incubated with 20 μM veratridine for 2 min at 35 °C, as previously described (Song and Marvizon, 2003a, 2005). Veratridine increases Na^+ fluxes through voltage-dependent Na^+ channels (Satoh and Nakazato, 1991), and evokes the release of enkephalin (Uzumaki et al., 1984) and dynorphin (Przewlocka et al., 1990) from the spinal cord. The evoked MOR internalization was maximal with veratridine concentrations of 10–30 μM and incubation times of 0.5–2 min. For the incubation, the slices were placed on a nylon net glued to a plastic ring inserted halfway down a plastic tube containing 5 ml aCSF. The aCSF was superficially gassed with a flow of 95% O_2/5% CO_2 passing through a needle inserted though the cap of the tube. To change solutions, the ring and net with the slice was transferred to a different tube. We previously found that MOR internalization induced by released opioids can only be observed if the opioids are protected from peptidase degradation (Song and Marvizon, 2003a). Accordingly, the slices were incubated with peptidase inhibitors (10 μM actinonin, captopril and thiorphan) starting 5 min before and ending 10 min after the addition of veratridine. Drugs tested in the experiments were added to the slices at the same time as the peptidase inhibitors, except when stated otherwise. After the 2 min incubation with veratridine, the slices were incubated for a further 10 min at 35 °C with peptidase inhibitors and other drugs to allow the completion of MOR internalization (Marvizon et al., 1999). At the end of the incubation the slices were fixed by immersion in ice-cold fixative (4% paraformaldehyde, 0.18% picric acid in 0.1 M sodium phosphate buffer).

2.3. Immunohistochemistry

Histological sections of 25 μm were cut from the slices and labeled as previously described (Marvizon et al., 1999; Song and Marvizon, 2003a; Song and Marvizon, 2003b, 2005). To label MORs we used a rabbit antiserum (1:7000 dilution) raised against amino acids 384–398 of the cloned rat MOR-1 (ImmunoStar, Hudson, WI, catalog no. 24216). This antiserum has been characterized (Arvidsson et al., 1995) and shown to label dorsal horn neurons (Spike et al., 2002). Pre-absorption of the MOR antibody with its immunizing peptide (10 μg/ml) abolished the staining. The secondary antibody was Alexa-488 goat anti-rabbit IgG (Molecular Probes, Eugene, OR), used at 1:2000 dilution for 2 h at room temperature. Sections were mounted in Prolong Gold with DAPI (Molecular Probes).

2.4. Quantification of MOR internalization

MOR internalization was quantified as previously described (Marvizon et al., 1999; Song and Marvizon, 2003a,b, 2005), by visually counting somata of MOR neurons while classifying them as with or without MOR internalization. This was done using a Zeiss Axio-Imager A1 (Carl Zeiss, Inc., Thornwood, NY) microscope with a fluorescence filter cube for Alexa Fluor 488 (excitation 460–500 nm, beam-splitter 505 nm, emission 510–560 nm; Chroma Technology Corporation, Rockingham, VT) and objectives of 63× (numerical aperture (NA) 1.40) and 100× (NA 1.40). Counting was done blind to the treatment. Neuronal somata with five or more endosomes were considered as having internalization. When necessary, the DAPI staining was used to confirm the presence of a cell soma. All MOR neurons of one dorsal horn were counted for each histological section, and 4 sections per slice (chosen randomly) were used. Typically, this amounted to 70–160 MOR neurons counted per slice. Data from one slice was considered as one replicate measure for statistical purposes.

2.5. Confocal microscopy

Confocal images were acquired at the UCLA's Carol Moss Spivak Cell Imaging Facility using a Leica TCS-SP confocal microscope (Leica Microsystems GmbH, Wetzlar, Germany). Excitation was provided by the 488 nm line of an argon laser, and the emission window was 500–560 nm. For this study we used a pinhole of 1.0 Airy unit and objectives of 20× (NA 0.7) and 100× oil (NA 1.4), resulting in an estimated optical section thickness

(full width at half maximum) of 2.53 and 0.62 μm, respectively. Images were acquired as confocal stacks with optical section separations (*z*-intervals) of 1.18 μm for the 20× objective and 0.203 μm for the 100× objective. Optical sections were averaged 3–4 times to reduce noise. Images were acquired at a digital size of 1024 × 1024 pixels, using a digital zoom of 2 with the 100× objective. They were later cropped to the relevant part of the field without changing the resolution. Images were processed using Imaris 5.0.2., 64-bit version (Bitplane AG, Saint Paul, MN, www.bitplane.com) and Adobe Photoshop 5.5 (Adobe Systems Inc., Mountain View, CA). Using Imaris, the confocal stacks were examined in 3-dimensions and cropped. For images obtained at 20×, the entire XY field and most of the optical sections were preserved. Images obtained at 100× were cropped in the XY dimension to the cell being examined, and in the *Z* dimension to optical sections through the middle of the cell. The resulting images were combined into a two-dimensional image and exported to Photoshop, which was used to adjust the contrast using its "adjustment layer/curves" feature, add text, and compose the multi-paneled figures.

2.6. Data analysis

Data were analyzed and graphics composed using Prism 5.1. (GraphPad Software, San Diego, CA). Data are given as the mean ± standard error (SE) of 3–18 slices. Statistical analyses consisted of one-way ANOVA and Bonferroni's post-test, with significance set at 0.05. The absence of asterisks in the figure indicates that the difference with control is not significant.

Time course data were fitted to a single phase exponential decay function: $Y = (Y_0 - P) \times \exp(X/\tau) + P$. Concentration–response data were fitted by a sigmoidal dose–response function: $Y = \text{bottom} + ((\text{top} - \text{bottom})/1 + 10^{(\log IC_{50} - \log X)})$, where "top" and "bottom" are the maximum and minimum values of the response (Y), respectively, and IC_{50} is the concentration (X) that produces half of the maximum inhibition. Parameter constraints were: top <100%, bottom >0%. We assumed that concentration–response curves were monotonic and sigmoidal. Baseline measures (zero concentration of drug) were included in the nonlinear regression by assigning them a concentration value about three log units lower than the IC_{50}. The statistical error of the IC_{50} was expressed as 95% confidence interval (CI). An *F*-test (Motulsky and Christopoulos, 2003) was used to determine which one of two functions with different number of parameters fitted the data better.

2.7. Chemicals

Actinonin, captopril, clonidine, endomorphin-2, thiorphan and veratridine were purchased from Sigma-RBI (St. Louis, MO). BRL-44408 (2-[2H-(1-methyl-1,3-dihydroisoindole)methyl]-4,5-dihydroimidazole maleate), guanfacine, JP-1302 (acridin-9-yl-[4-(4-methylpiperazin-1-yl)-phenyl]amine trihydrochloride hydrate), medetomidine (4-[1-(2,3-dimethylphenyl)ethyl]-1*H*-imidazole hydrochloride), UK-14304 (brimonidine, 5-bromo-*N*-(4,5-dihydro-1*H*-imidazol-2-yl)-6-quinoxalinamine) and rauwolscine were purchased from Tocris (Ellisville, MO). Isoflurane was from Halocarbon Laboratories, River Edge, NJ.

3. Results

3.1. MOR internalization evoked by veratridine

To evoke the release of opioid peptides, we incubated rat spinal cord slices with 20 μM veratridine for 2 min at 35 °C. Veratridine increases neuronal firing by opening voltage-gated Na^+ channels (Satoh and Nakazato, 1991), and has been shown to induce opioid release in the spinal cord (Chen et al., in press; Song and Marvizon, 2003a, 2005; Uzumaki et al., 1984). Opioid release was measured in situ by assessing the internalization of MORs in the somata of dorsal horn neurons, as shown in Fig. 1. Veratridine induced the redistribution of MOR immunoreactivity from the cell surface to small particles (endosomes) located inside the cell but outside the nucleus (Fig. 1A). We have shown previously (Song and Marvizon, 2003a) that this veratridine-evoked MOR internalization is due to the release of opioids that bind to the MOR, because it was eliminated by the selective MOR antagonist CTAP and substantially reduced in the presence of low concentrations of Ca^{2+} and in the absence of peptidase inhibitors. To inhibit the enkephalin-cleaving peptidases aminopeptidase N, dipeptidyl carboxypeptidase and neutral endopeptidase, we included in the incubation media their respective inhibitors actinonin, captopril and thiorphan, all at a concentration of 10 μM. Inhibiting this enzymes is required to observe MOR internalization induced by opioid peptides, either released endogenously or added exogenously (Chen et al., 2007b; Song and Marvizon, 2003a). Peptidase inhibitors by themselves do not induce MOR internalization (Chen et al., 2007b; Song and Marvizon, 2003a). In these conditions, veratridine induced MOR internalization in 71 ± 2% ($n = 39$ slices) of the MOR-immunoreactive neurons in the dorsal horn. The amount of evoked MOR internalization could not be increased by incubating the slices with higher concentrations of veratridine or for longer time periods. Quite the opposite: 100 μM veratridine directly inhibits the MOR internalization mechanism itself (Chen et al., in press).

3.2. Adrenergic α_2 agonists inhibited veratridine-evoked MOR internalization

To determine whether adrenergic α_2 receptors inhibit opioid release in the spinal cord, we investigated the ability of four α_2 agonists to inhibit veratridine-evoked MOR internalization. All agonists inhibited the evoked MOR internalization. Slices were preincubated with the agonists (and peptidase inhibitors) for 5 min before veratridine stimulation. As can be observed in Fig. 2, shorter preincubation times (1 or 0 min before veratridine) resulted in the loss of the inhibition by the agonist guanfacine (3 μM), whereas a longer preincubation time (10 min) produced the same result as 5 min. This probably reflects the time required for the drugs to penetrate the slices. After veratridine, slices were incubated for a further 10 min to allow the completion of the internalization process (Marvizon et al., 1999).

The classic α_2 agonist clonidine (Fig. 3A) produced an almost complete inhibition of the evoked MOR internalization. Its effect follows a single phase concentration–response curve with an IC_{50} of 1.7 μM ($\log IC_{50} = -5.76 \pm 0.16$). This value was higher than the potency of clonidine to displace [^{3}H]MK 912 binding from rat cerebral cortex (Table 1), perhaps reflecting a difference between α_2 receptors between the brain and the spinal cord.

Guanfacine, another α_2 adrenergic agonist, also produced a practically complete inhibition of MOR internalization (Fig. 3B). Its potency was almost one order of magnitude higher than clonidine, with an IC_{50} of 248 nM ($\log IC_{50} = -6.61 \pm 0.13$), a value consistent with its affinity for α_{2C} receptors (but not for α_{2A} receptors, Table 1). Fig. 1C

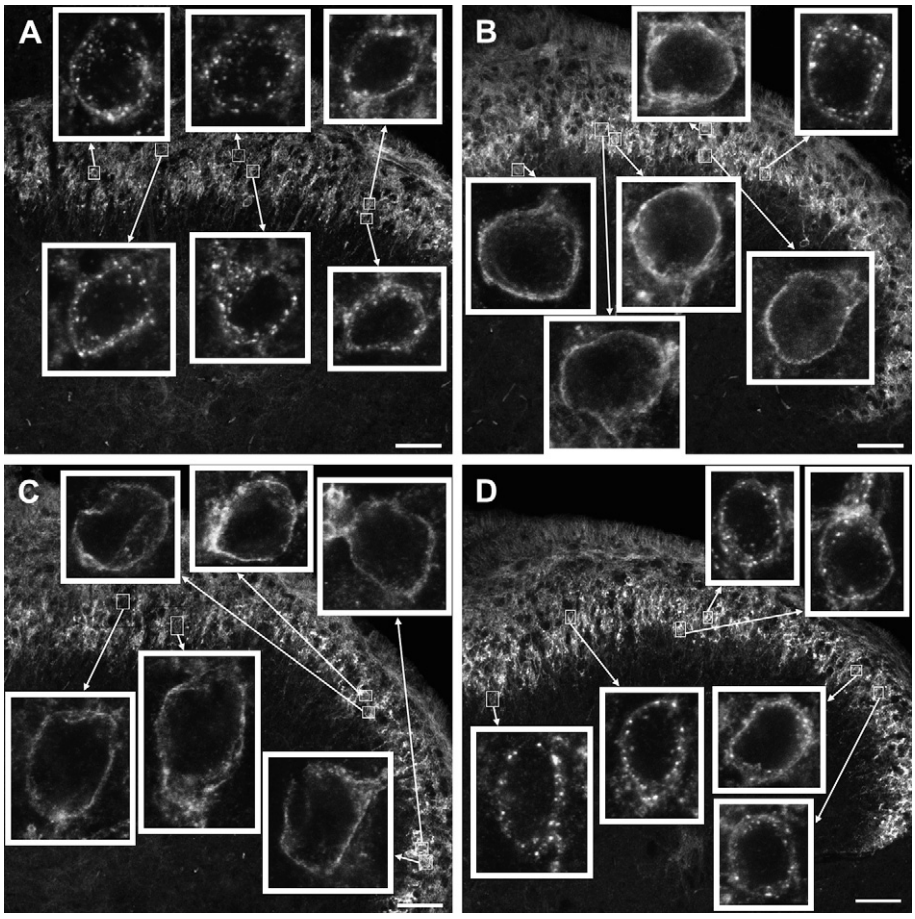

Fig. 1. Images of MOR neurons in the dorsal horn after evoking opioid release with veratridine. Spinal cord slices were incubated at 35 °C with 20 μM veratridine, peptidase inhibitors (actinonin, captopril and thiorphan, 10 μM) and the following α_2 adrenergic compounds: (A) none; (B) the agonist UK-14,304 (30 nM); (C) the agonist guanfacine (3 μM); (D) guanfacine (3 μM) and the antagonist JP-1203 (3 nM). Opioid release resulted in the internalization of MORs in dorsal horn neurons, visualized with immunofluorescence. Abundant MOR internalization is observed in the control (A) and after guanfacine plus the antagonist JP-1203 (D), but little internalization was evoked in the presence of the agonists UK-14,304 (B) or guanfacine (C). Main panels show most of the dorsal horn: images were taken with a 20× objective (scale bars are 50 μm) and consist of 4 confocal sections separated 1.18 μm taken trough the middle of the histological section. Insets show examples of MOR neurons: images were taken with a 100× objective and a digital zoom of 2 (scale bars are 5 μm) and consist of 4–12 optical sections spaced 0.20 μm taken through the middle of the cells.

shows examples of dorsal horn neurons in a slice stimulated with veratridine in the presence of 3 μM guanfacine: MOR immunoreactivity is present at the cell surface and endosomes are absent from the cytoplasm.

A third α_2 agonist, medetomidine, was also studied. Medetomidine is the racemic form of dexmedetomidine, which is used clinically. Fig. 3C shows that medetomidine inhibited the evoked MOR internalization with very high potency ($IC_{50} = 0.3$ nM, $\log IC_{50} = -9.50 \pm 0.36$) consistent with previous reports (Table 1). However, medetomidine inhibited the evoked MOR internalization only partially: the inhibition reached a plateau at $37 \pm 5\%$ MOR neurons with internalization. These results indicate that medetomidine behaves as a partial agonist of the α_2 receptor that inhibits opioid release. Alternatively, the effect of medetomidine can be interpreted as an initial inhibition phase followed by a reversal of the inhibition at concentrations higher then 3 nM, as indicated by the dotted line in Fig. 3 C (“bell-shaped” curve). However, comparing the fittings to the two functions (i.e., single phase inhibition vs. inhibition followed by reversal) with an F-test revealed that the single phase inhibition provided a better fit of the data ($p = 0.279$).

A fourth α_2 agonist, UK-14304 (brimonidine), produced an even more complex effect on the evoked MOR internalization (Fig. 2D): an inhibitory phase up to 30 nM, followed by reversal of the inhibition at 50 and 100 nM, and a second inhibition phase at 300 nM and 1 μM. It was not possible to fit all the points to a single function. In view of this, we tentatively fitted the points up to 30 nM to a concentration–response curve, obtaining an approximate IC_{50} of 22 nM ($\log IC_{50} = -7.66 \pm 0.46$) for the initial inhibitory phase. This would reflect a potency 10-times higher than the one reported in the brain (Table 1). The complex effect of UK-14304 may be caused by its interaction with receptors other than α_2 adrenergic receptors at its higher concentrations. Fig. 1B illustrates the inhibition by 30 nM UK-1403 of MOR internalization evoked by veratridine: in five of the six MOR neurons shown MOR immunoreactivity is present at the cell surface and not in endosomes.

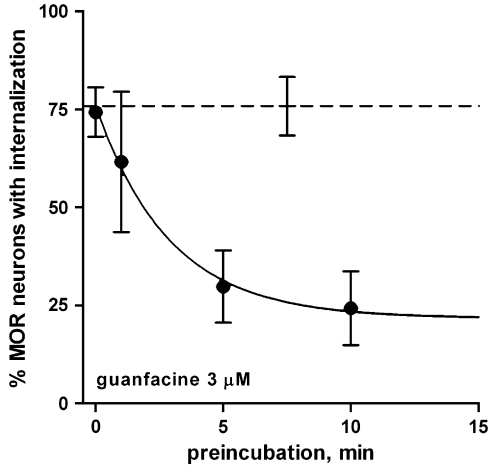

Fig. 2. Effect of preincubation time with guanfacine. Spinal cord slices were pre-incubated for 10 min with peptidase inhibitors (actinonin, captopril and thiorphan, 10 μM), and with 3 μM guanfacine for the pre-incubation times indicated on the X-axis. Then, opioid release was evoked with 20 μM veratridine for 2 min. Slices were kept in aCSF containing peptidase inhibitors for 10 more minutes before fixing. The dotted line indicates the amount of MOR internalization obtained in the absence of guanfacine. The curve represents the fit of the points to an exponential decay function (half-life 2 min).

3.3. Adrenergic α_2 agonists did not inhibit MOR internalization induced by a MOR agonist

Since MOR internalization evoked by veratridine was due to opioid release, we attributed its inhibition by α2 agonists to an inhibition of opioid release mediated by α2 adrenergic receptors. However, the α2 agonists could have inhibited the MOR internalization process itself. To rule out this possibility, we induced MOR internalization by incubating the slices with the MOR agonist endomorphin-2 in the absence and presence of α2 agonists. Slices were incubated with the α2 agonists for 5 min at 35 °C, and then 100 nM endomorphin-2 was added for another 10 min. No peptidase inhibitors were used, because endomorphin-2 is not appreciably degraded by peptidases in these conditions (Song and Marvizon, 2003a). In agreement with our previous results (Song and Marvizon, 2003a), endomorphin-2 induced MOR internalization in most MOR dorsal horn neurons (Fig. 4). The internalization induced by endomorphin-2 was not affected by 3 μM clonidine, 3 μM guanfacine, 3 nM medetomidine, or 30 nM UK-14304, their most effective concentrations to inhibit of veratridine-evoked MOR internalization (Fig. 3). Therefore, the inhibition of veratridine-evoked MOR internalization by α2 agonists should be attributed to inhibition of opioid release and not to inhibition of MOR internalization itself.

3.4. Reversal by α_{2C} antagonists of the inhibition of MOR internalization by α_2 agonists

There are three pharmacologically and genetically distinct subtypes of α_2 adrenergic receptors: α_{2A}, α_{2B} and α_{2C} (Yaksh, 1985). The α_{2C} subtype has been found to colocalize with opioids in presynaptic terminals of intrinsic dorsal horn neurons (Marvizon et al., 2007; Olave and Maxwell, 2002, 2004; Stone et al., 1998) and therefore is likely to mediate the inhibition of opioid release produced by the α_2 agonists. To investigate this possibility, we determined whether the subtype-selective α_{2C} antagonist JP-1203 (Sallinen et al., 2007; Tricklebank, 2007) reversed the inhibition produced by the agonists. There are no subtype-selective α_2 agonists available. Although guanfacine shows some selectivity for α_{2A} receptors (Table 1), its relatively high IC_{50} in Fig. 3B suggests that its effect is mediated by α_{2C} receptors. The antagonist JP-1302 has a 100-fold selectivity for α_{2C} versus α_{2A} receptors (Table 1). Its reported K_b (Table 1) to antagonize human α_{2C} receptors is 16 nM (Table 1), but we found that in our preparation it was fully effective at a lower concentration (10 nM) to reverse the inhibitions produced by 3 μM clonidine, 3 μM guanfacine and 30 nM UK-14304 (Fig. 5). JP-1302 did not have any effect by itself. Fig. 1D shows representative dorsal horn neurons in a slice incubated with guanfacine (3 μM) and JP-1302 (3 nM). Note that MOR internalization is abundant in all neurons, whereas no MOR internalization is observed in a slice incubated with 3 μM guanfacine alone (Fig. 1C). In contrast, the antagonist BRL-44408 at 100 nM, a concentration, at which it blocks α_{2A} receptors (Jurgens et al., 2007) but not α_{2C} receptors (Table 1), did not prevent the inhibition produced by 3 μM guanfacine (Fig. 5). The antagonist rauwolscine at 30 nM, a concentration at which it should block α_{2C} receptors but only partially block α_{2A} receptors (Table 1), also abolished the inhibition produced by 30 nM UK-14304. These results show that the inhibition of the evoked MOR internalization produced by guanfacine, clonidine and low concentrations of UK-14304 is caused by the activation of α_{2C} receptors.

To determine whether the second inhibition phase produced by UK-14304 at concentrations higher than 100 nM is mediated by α_2 receptors of other receptors, we studied whether it was blocked by rauwolscine (Fig. 6). Rauwolscine partially prevented the inhibition by 1 μM UK-14304 at 1 μM and abolished it at 10 μM. This indicates that the second inhibition produced by UK-14304 is also mediated by α_2 receptors. The need for high concentrations of rauwolscine further suggests that rauwolscine antagonizes the effect of UK-14304 in a competitive fashion. This requirement for high concentrations of antagonists prevented the determination of the α_2 subtype that mediates the second inhibitory phase of UK-14304.

4. Discussion

Our results demonstrate for the first time the existence of an adrenergic inhibition of opioid release in the spinal dorsal horn, mediated by α_{2C} adrenergic receptors.

4.1. Mechanism

We show that four agonists of α_2 adrenergic receptors—clonidine, guanfacine, medetomidine and UK-14304—inhibited MOR internalization evoked by veratridine. Since these four agonists did not affect MOR internalization produced by the MOR agonist endomorphin-2, their effect has to

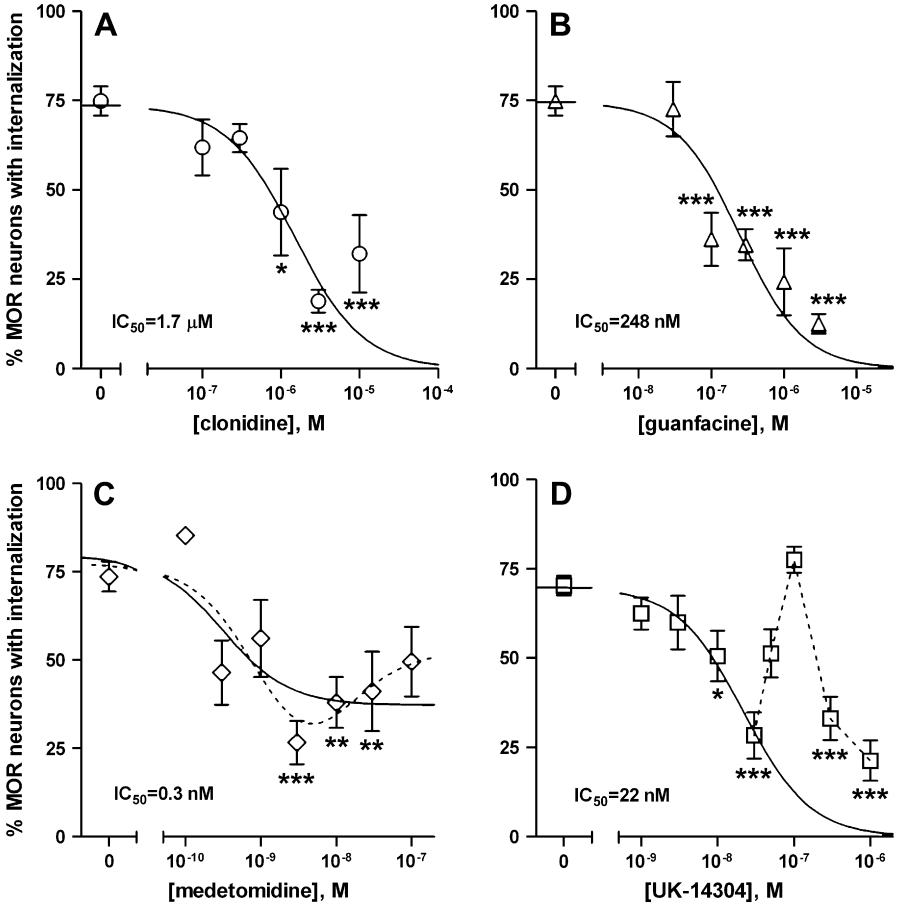

Fig. 3. Inhibition of opioid release by adrenergic α_2 agonists. Opioid release and subsequent MOR internalization was evoked by stimulating spinal cord slices with 20 μM veratridine in the presence of peptidase inhibitors (actinonin, captopril and thiorphan, 10 μM). Concentration–response curves were obtained for the α_2 adrenergic agonists clonidine (A), guanfacine (B), medetomidine (C), and UK-14304 (D). Data points give the mean ± SEM of 3–18 slices. ANOVA and Bonferroni's post-test were used to determine whether there were significant differences with the controls (zero concentration of agonist): $^*p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$. Curves represent the fit of the data to a sigmoidal dose–response function. (A) Clonidine produced a total inhibition with an IC_{50} of 1.7 μM (95% CI 0.8–3.7 μM). (B) Guanfacine produced a total inhibition with an IC_{50} of 248 nM (95% CI 131–67 nM). (C) Medetomidine produced a partial inhibition that leveled off at 37 ± 5%, with an IC_{50} of 0.3 nM (95% CI 0.06–1.7 μM). The dotted line represents the alternative fit of the data to a bell-shaped curve: inhibition ($\log IC_{50} = -8.96 \pm 0.84$) followed by reversal of the inhibition ($\log EC_{50} = -8.02 \pm 1.53$). However, an F-test revealed that the bell-shaped curve did not provide a better fit of the data ($p = 0.279$). (D) UK-14304 produced a complex dose–response consisting of an inhibition phase up to 30 nM followed by reversal of the inhibition at 100 nM and a second inhibition phase. Fitting the points up to 30 nM UK-14304 to a dose–response function yielded an IC_{50} of 22 nM (95% CI 2.5–189 nM). Points at higher concentrations are linked by a dotted line.

be attributed to an inhibition of the opioid release evoked by veratridine. The inhibition produced by the agonists was prevented by the selective α_{2C} antagonist JP-1302, but not by the α_{2A} antagonist BRL-44408. Therefore, the inhibition of opioid release was caused by the activation of α_{2C} receptors.

This conclusion is consistent with previous immunohistochemistry studies demonstrating a substantial colocalization in the dorsal horn of α_{2C} receptors with Met-enkephalin (Olave and Maxwell, 2002, 2004), Leu-enkephalin (Stone et al., 1998) or the pan-opioid antibody 3-E7 (Marvizon et al., 2007). In contrast, α_{2C} receptors did not colocalize with preprodynorphin (Stone et al., 1998), which appears to be expressed in dorsal horn neurons different from the ones expressing preproenkephalin (Cruz and Basbaum, 1985). Therefore, the decrease in evoked MOR internalization produced by α_{2C} agonists was probably caused by an inhibition of enkephalin release, and not of dynorphin release. Substance P-containing primary afferent terminals in the dorsal horn have been reported to contain the putative endogenous opioids endomorphin-1 and -2, based on immunohistochemistry studies (Martin-Schild et al., 1998; Nydahl et al., 2004). These terminals also have α_{2A} adrenergic receptors (Stone et al., 1998). However, our previous studies established that the opioids that are released in the dorsal horn to induce MOR internalization are not endomorphins. First, the opioids that induce MOR internalization are very susceptible to peptidase degradation, while endomorphins are resistant to peptidase degradation (Song and Marvizon, 2003a). Second, electrical stimulation of the dorsal root did not produce MOR internalization, even though it produced widespread neurokinin 1 receptor internalization by releasing substance P from primary afferent terminals (Song and Marvizon, 2003b). This is consistent with our finding that opioid release is inhibited by the α_{2C} receptors found in interneurons, and not by the α_{2A} receptors found in primary afferent terminals.

Table 1
Adrenergic α_2 compounds used in the study

Compound	Potency α_{2A} (nM)	Potency α_{2C} (nM)	Reference
Agonists			
Clonidine	73	81	Uhlen et al., 1992
Guanfacine	19	421	Uhlen et al., 1992
Medetomidine	1[a]	1[a]	Virtanen et al., 1988
UK-14304 (brimonidine)	277	221	Uhlen et al., 1992
Antagonists			
BRL-44408	14	128	Uhlen et al., 1992
JP-1302	1500	16	Sallinen et al., 2007
Rauwolscine	34	1.6	Uhlen et al., 1992

The second and third columns indicate the approximate potencies (K_i or K_b) of the compounds for α_{2A} and α_{2C} receptors, respectively. Data from Uhlen et al. (1992) correspond to displacement of [^{3}H]MK 912 binding from rat cerebral cortex. Data from Sallinen et al. (2007) represent antagonist activity (K_b) on adrenaline-induced stimulation of [^{35}S]GTPγS binding to Chinese hamster ovary cells expressing human α_{2A} or α_{2C} receptors. Data from Virtanen et al. (1988) correspond to displacement of [^{3}H]clonidine binding from rat brain membranes.

[a] Subtype selectivity not determined.

The presence of α_{2C} receptors in enkephalin-containing terminals suggests that they inhibit enkephalin release using an intracellular signaling pathway inside the presynaptic terminal. Adrenergic α_2 receptors signal through inhibitory α_i G proteins (Summers and McMartin, 1993) and inhibit the release of other neurotransmitters (Li and Eisenach, 2001). Therefore, the most likely mechanism for the inhibition of release would be the inactivation of voltage-gated Ca^{2+} channels

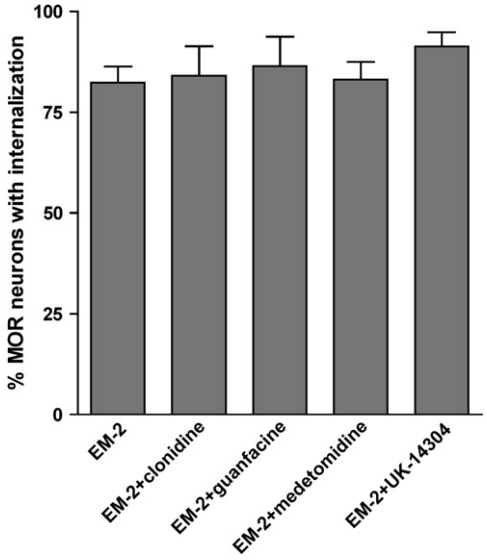

Fig. 4. Adrenergic α_2 agonists did not inhibit MOR internalization induced by endomorphin-2. MOR internalization was evoked by stimulating spinal cord slices with the MOR agonist endomorphin-2 (100 nM) for 10 min. The α_2 agonists were added 5 min prior to endomorphin-2 at concentrations that produced maximum inhibition of veratridine-evoked MOR internalization: clonidine 3 μM, guanfacine 3 μM, medetomidine 3 nM, UK-14304 30 nM. ANOVA revealed no significant differences with endomorphin-2 alone ($p = 0.79$).

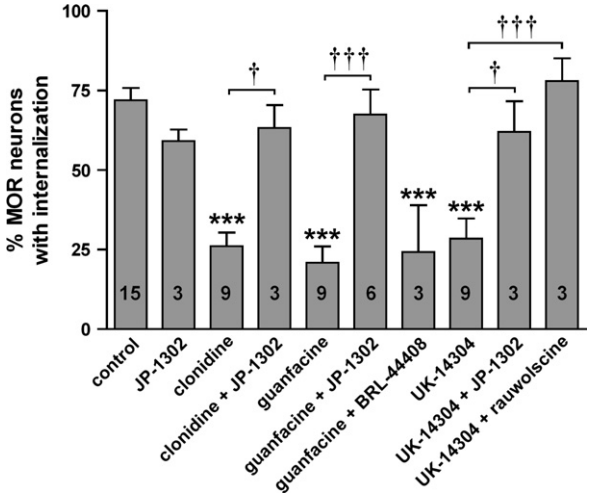

Fig. 5. Inhibitions of evoked MOR internalization by clonidine, guanfacine or UK-14304 were prevented by the α_{2C} antagonist JP-1302. Opioid release and subsequent MOR internalization was evoked by stimulating spinal cord slices with 20 μM veratridine in the presence of peptidase inhibitors (actinonin, captopril and thiorphan, 10 μM). Clonidine (3 μM), guanfacine (3 μM) or UK-14304 (30 nM) inhibited the evoked MOR internalization. The selective α_{2C} antagonist JP-1302 (10 nM), applied together with clonidine, guanfacine or UK-14304, prevented the inhibition of MOR internalization. JP-1302 did not have any effect by itself. The selective α_{2A} antagonist BRL-44408 (100 nM) did not reverse the inhibition produced by guanfacine. Inhibition by UK-14304 was also prevented by the α_2 antagonist rauwolscine (30 nM). Numbers inside the bars indicated the number of slices (n) in each group. Statistical significance was determined with one-way ANOVA (overall $p < 0.0001$) and Bonferroni's post-test: $^{***}p < 0.001$ compared to control; $^{\dagger\dagger\dagger}p < 0.001$, $^{\dagger}p < 0.05$, for the comparisons indicated.

by the βγ subunits of G proteins (Adamson et al., 1989; Dolphin, 2003; Li and Bayliss, 1998). However, our experimental approach cannot completely rule out that the adrenergic agonists acted on sites other than the opioid-containing presynaptic terminals.

4.2. Physiological role

Opioids released in the dorsal horn produce analgesia, mainly by activating MORs (Budai and Fields, 1998; Chen et al., 2007b; Takemori and Portoghese, 1993). Therefore, in situations where endogenous opioids are producing some level of analgesia, the inhibition of opioid release by α_{2C} receptors would be expected to increase pain. This seems to run contrary to the well-established fact that α_2 adrenergic receptors in the spinal cord produce analgesia (Pertovaara, 2006; Yaksh, 1985). This analgesia is largely mediated by the α_{2A} subtype of adrenergic receptors, which inhibit the release of glutamate and substance P from primary afferent terminals (Kamisaki et al., 1993; Kawasaki et al., 2003; Kuraishi et al., 1985; Li and Eisenach, 2001; Pan et al., 2002; Takano et al., 1993). However, there is also evidence that α_{2C} receptors contribute to this analgesic effect (Fairbanks et al., 2002).

Hence, these α_{2C} receptors may play a complex role in modulation of pain in the dorsal horn. As stated above, α_{2C} receptors and enkephalins are present together in the presynaptic

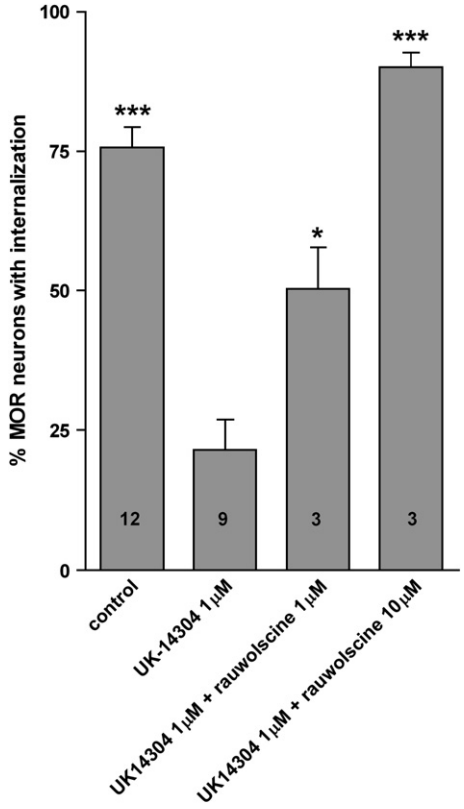

Fig. 6. The second inhibition phase of UK-14304 was prevented by rauwolscine. MOR internalization was evoked by stimulating spinal cord slices with 20 μM veratridine in the presence of peptidase inhibitors (actinonin, captopril and thiorphan, 10 μM). At 1 μM, UK-14304 produced a second inhibition phase of the evoked MOR internalization. The α_2 antagonist rauwolscine partially prevented this inhibition at 1 μM and abolished it at 10 μM. Numbers inside the bars indicated the number of slices (n) in each group. Statistical significance was determined with one-way ANOVA (overall $p < 0.0001$) and Bonferroni's post-test: $^{***}p < 0.001$ compared to UK-14304.

terminals of dorsal horn interneurons (Olave and Maxwell, 2002, 2004; Stone et al., 1998). Paradoxically, however, the majority of these interneurons are excitatory, and make glutamatergic synapses with nociceptive projection neurons (Olave and Maxwell, 2003a,b). Therefore, despite the fact that they release enkephalins, these interneurons seem to belong to a pro-algesic pathway. It is likely that the α_{2C} receptors, besides inhibiting enkephalin release, also inhibit glutamate release. The resulting decrease in the excitatory input to the projection neurons may be responsible for the analgesic effect of the α_{2C} receptors.

Then, what could be the physiological role of the inhibition of enkephalin release by α_{2C} adrenergic receptors? Noradrenergic terminals in the dorsal horn belong to descending axons that originate in adrenergic nuclei of the brain stem (Pertovaara, 2006). These terminals do not appear to form synapses (Rajaofetra et al., 1992), which implies that norepinephrine acts by volume transmission in the dorsal horn (Zoli and Agnati, 1996). Therefore, whenever norepinephrine is released in the dorsal horn, it would concurrently produce analgesia by acting on α_{2A} receptors and inhibit enkephalin release by acting on α_{2C} receptors. This could serve to shut down the spinal opioid system whenever the spinal adrenergic system is active. This may be a way to avoid the excessive analgesia that could ensue if both the adrenergic and the opioid systems in the spinal cord are active at the same time.

4.3. Relationship with other neurotransmitter receptors that modulate spinal opioid release

Adrenergic α_{2C} receptors are in no way unique in their ability to inhibit opioid release. In a previous study (Song and Marvizon, 2005), we showed that spinal opioid release is inhibited by NMDA receptors acting in conjunction with large conductance Ca^{2+}-sensitive K^+ (BK) channels. It is likely that these NMDA receptors and BK channels are extrasynaptic (Isaacson and Murphy, 2001), and not presynaptic like the α_{2C} receptors. Therefore, these two modulatory mechanisms probably act independently.

More recently, we showed that spinal opioid release is inhibited by serotonin 5-HT_{1A} receptors (Song et al., 2007). However, the 5-HT_{1A} agonist 8-hydroxy-DPAT inhibited only half of the evoked MOR internalization, suggesting that these receptors inhibit release from only some of the opioid terminals. Alternatively, inhibition by 5-HT_{1A} receptors may require an extracellular signal. In contrast, we found that opioid release is not affected by $GABA_A$, $GABA_B$, cholecystokinin or δ-opioid receptors (Song and Marvizon, 2005). A substantial challenge for future studies will be to explain how these different neurotransmitter systems interact with each other to modulate spinal opioid release.

4.4. Therapeutic implications

It may be possible to produce analgesia by increasing the availability of opioid peptides in the spinal cord. In fact, blocking peptidase degradation of opioids produced analgesia (Chou et al., 1984; Fournie-Zaluski et al., 1992; Noble et al., 1992b), with the added benefit of producing little tolerance and dependence (Noble et al., 1992a,c). Another way to increase the availability of opioids in the spinal cord would be by blocking neurotransmitter receptors that inhibit their release. However, as explained above, blocking α_{2C} receptors is unlikely to produce analgesia because the disinhibition of glutamate release onto nociceptive neurons would probably cancel the analgesic effect produced by the increase in opioid release. Something different may happen in situations like inflammation, where there is an increased activity of descending noradrenergic pathways (Pertovaara, 2006). In these cases inhibiting α_{2C} receptors would increase the release of opioids, which then would act synergistically with α_{2A} receptors (Monasky et al., 1990; Ossipov et al., 1990; Stone et al., 1997) to increase the analgesia. Further studies using in vivo pain models are needed to address this issue.

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Appendix A. Supplementary material

Supplementary data associated with this article can be found in the online version, at doi:10.1016/j.neuropharm.2008.02.002.

References

Adamson, P., Xiang, J.Z., Mantzourides, T., Brammer, M.J., Campbell, I.C., 1989. Presynaptic alpha 2-adrenoceptor and kappa-opiate receptor occupancy promotes closure of neuronal (N-type) calcium channels. European Journal of Pharmacology 174, 63–70.

Arvidsson, U., Riedl, M., Chakrabarti, S., Lee, J.H., Nakano, A.H., Dado, R.J., Loh, H.H., Law, P.Y., Wessendorf, M.W., Elde, R., 1995. Distribution and targeting of a mu-opioid receptor MOR1 in brain and spinal cord. Journal of Neuroscience 15, 3328–3341.

Bohn, L.M., Xu, F., Gainetdinov, R.R., Caron, M.G., 2000. Potentiated opioid analgesia in norepinephrine transporter knock-out mice. Journal of Neuroscience 20, 9040–9045.

Budai, D., Fields, H.L., 1998. Endogenous opioid peptides acting at mu-opioid receptors in the dorsal horn contribute to midbrain modulation of spinal nociceptive neurons. Journal of Neurophysiology 79, 677–687.

Cesselin, F., Bourgoin, S., Clot, A.M., Hamon, M., Le Bars, D., 1989. Segmental release of met-enkephalin-like material from the spinal-cord of rats, elicited by noxious thermal stimuli. Brain Research 484, 71–77.

Chen, S.R., Pan, H.L., 2006. Blocking mu opioid receptors in the spinal cord prevents the analgesic action by subsequent systemic opioids. Brain Research 1081, 119–125.

Chen, W., Marvizon, J.C., Song, B., 2007a. Inhibition of opioid release in the rat spinal cord by alpha-2 adrenergic receptors. Society for Neuroscience Abstracts 36 922.921.

Chen, W., Song, B., Lao, L., Perez, O.A., Kim, W., Marvizon, J.C.G., 2007b. Comparing analgesia and μ-opioid receptor internalization produced by intrathecal enkephalin: requirement for peptidase inhibition. Neuropharmacology 53, 664–667.

Chen, W., Song, B., Zhang, G., Marvizon, J.C. Effects of veratridine and high potassium on μ-opioid receptor internalization in the rat spinal cord: stimulation of opioid release versus inhibition of internalization. Journal of Neuroscience Methods, in press.

Chou, J., Tang, J., Del Rio, J., Yang, H.Y., Costa, E., 1984. Action of peptidase inhibitors on methionine5-enkephalin-arginine6-phenylalanine7 (YGGFMRF) and methionine5-enkephalin (YGGFM) metabolism and on electroacupuncture antinociception. Journal of Pharmacology and Experimental Therapeutics 230, 349–352.

Cruz, L., Basbaum, A.I., 1985. Multiple opioid peptides and the modulation of pain: immunohistochemical analysis of dynorphin and enkephalin in the trigeminal nucleus caudalis and spinal cord of the cat. Journal of Comparative Neurology 240, 331–348.

Dolphin, A.C., 2003. G protein modulation of voltage-gated calcium channels. Pharmacological Reviews 55, 607–627.

Eckersell, C.B., Popper, P., Micevych, P.E., 1998. Estrogen-induced alteration of μ-opioid receptor immunoreactivity in the medial preoptic nucleus and medial amygdala. Journal of Neuroscience 18, 3967–3976.

Fairbanks, C.A., Stone, L.S., Kitto, K.F., Nguyen, H.O., Posthumus, I.J., Wilcox, G.L., 2002. alpha(2C)-adrenergic receptors mediate spinal analgesia and adrenergic-opioid synergy. Journal of Pharmacology and Experimental Therapeutics 300, 282–290.

Fournie-Zaluski, M.C., Coric, P., Turcaud, S., Lucas, E., Noble, F., Maldonado, R., Roques, B.P., 1992. "Mixed inhibitor–prodrug" as a new approach toward systemically active inhibitors of enkephalin-degrading enzymes. Journal of Medicinal Chemistry 35, 2473–2481.

Isaacson, J.S., Murphy, G.J., 2001. Glutamate-mediated extrasynaptic inhibition: direct coupling of NMDA receptors to $Ca^{(2+)}$-activated K^+ channels. Neuron 31, 1027–1034.

Jensen, T.S., Yaksh, T.L., 1984. Spinal monoamine and opiate systems partly mediate the antinociceptive effects produced by glutamate at brainstem sites. Brain Research 321, 287–297.

Jurgens, C.W., Hammad, H.M., Lichter, J.A., Boese, S.J., Nelson, B.W., Goldenstein, B.L., Davis, K.L., Xu, K., Hillman, K.L., Porter, J.E., Doze, V.A., 2007. α2A adrenergic receptor activation inhibits epileptiform activity in the rat hippocampal CA3 region. Molecular Pharmacology 71, 1572–1581.

Kamisaki, Y., Hamada, T., Maeda, K., Ishimura, M., Itoh, T., 1993. Presynaptic alpha 2 adrenoceptors inhibit glutamate release from rat spinal cord synaptosomes. Journal of Neurochemistry 60, 522–526.

Kawasaki, Y., Kumamoto, E., Furue, H., Yoshimura, M., 2003. Alpha 2 adrenoceptor-mediated presynaptic inhibition of primary afferent glutamatergic transmission in rat substantia gelatinosa neurons. Anesthesiology 98, 682–689.

Kuraishi, Y., Hirota, N., Sato, Y., Kaneko, S., Satoh, M., Takagi, H., 1985. Noradrenergic inhibition of the release of substance P from the primary afferents in the rabbit spinal dorsal horn. Brain Research 359, 177–182.

Le Bars, D., Bourgoin, S., Clot, A.M., Hamon, M., Cesselin, F., 1987a. Noxious mechanical stimuli increase the release of Met-enkephalin-like material heterosegmentally in the rat spinal cord. Brain Research 402, 188–192.

Le Bars, D., Bourgoin, S., Villanueva, L., Clot, A.M., Hamon, M., Cesselin, F., 1987b. Involvement of the dorsolateral funiculi in the spinal release of met-enkephalin-like material triggered by heterosegmental noxious mechanical stimuli. Brain Research 412, 190–195.

Li, X., Eisenach, J.C., 2001. alpha2A-Adrenoceptor stimulation reduces capsaicin-induced glutamate release from spinal cord synaptosomes. Journal of Pharmacology and Experimental Therapeutics 299, 939–944.

Li, Y.W., Bayliss, D.A., 1998. Activation of alpha 2-adrenoceptors causes inhibition of calcium channels but does not modulate inwardly-rectifying K^+ channels in caudal raphe neurons. Neuroscience 82, 753–765.

Martin-Schild, S., Gerall, A.A., Kastin, A.J., Zadina, J.E., 1998. Endomorphin-2 is an endogenous opioid in primary sensory afferent fibers. Peptides 19, 1783–1789.

Marvizon, J.C., Grady, E.F., Waszak-McGee, J., Mayer, E.A., 1999. Internalization of μ-opioid receptors in rat spinal cord slices. Neuroreport 10, 2329–2334.

Marvizon, J.C., Pérez, O.A., Song, B., Chen, W., Bunnett, N.W., Grady, E.F., Todd, A.J., 2007. Calcitonin receptor-like receptor and receptor activity modifying protein 1 in the rat dorsal horn: localization in glutamatergic presynaptic terminals containing opioids and adrenergic α2C receptors. Neuroscience 148, 250–265.

Mills, R.H., Sohn, R.K., Micevych, P.E., 2004. Estrogen-induced μ-opioid receptor internalization in the medial preoptic nucleus is mediated via neuropeptide Y-Y1 receptor activation in the arcuate nucleus of female rats. Journal of Neuroscience 24, 947–955.

Monasky, M.S., Zinsmeister, A.R., Stevens, C.W., Yaksh, T.L., 1990. Interaction of intrathecal morphine and ST-91 on antinociception in the rat: dose–response analysis, antagonism and clearance. Journal of Pharmacology and Experimental Therapeutics 254, 383–392.

Morgan, M.M., Gold, M.S., Liebeskind, J.C., Stein, C., 1991. Periaqueductal gray stimulation produces a spinally mediated, opioid antinociception for the inflamed hindpaw of the rat. Brain Research 545, 17–23.

Motulsky, H., Christopoulos, A., 2003. Fitting Models to Biological Data Using Linear and Nonlinear Regression. GraphPad Software, Inc., San Diego, CA.

Noble, F., Coric, P., Fournie-Zaluski, M.C., Roques, B.P., 1992a. Lack of physical dependence in mice after repeated systemic administration of the mixed inhibitor prodrug of enkephalin-degrading enzymes, RB101. European Journal of Pharmacology 223, 91–96.

Noble, F., Soleilhac, J.M., Soroca-Lucas, E., Turcaud, S., Fournie-Zaluski, M.C., Roques, B.P., 1992b. Inhibition of the enkephalin-metabolizing enzymes by the first systemically active mixed inhibitor prodrug RB 101 induces potent analgesic responses in mice and rats. Journal of Pharmacology and Experimental Therapeutics 261, 181–190.

Noble, F., Turcaud, S., Fournie-Zaluski, M.C., Roques, B.P., 1992c. Repeated systemic administration of the mixed inhibitor of enkephalin-degrading enzymes, RB101, does not induce either antinociceptive tolerance or cross-tolerance with morphine. European Journal of Pharmacology 223, 83–89.

North, R.A., Yoshimura, M., 1984. The actions of noradrenaline on neurones of the rat substantia gelatinosa in vitro. Journal of Physiology 349, 43–55.

Nydahl, K.S., Skinner, K., Julius, D., Basbaum, A.I., 2004. Co-localization of endomorphin-2 and substance P in primary afferent nociceptors and effects of injury: a light and electron microscopic study in the rat. European Journal of Neuroscience 19, 1789–1799.

Olave, M.J., Maxwell, D.J., 2002. An investigation of neurones that possess the alpha 2C-adrenergic receptor in the rat dorsal horn. Neuroscience 115, 31–40.

Olave, M.J., Maxwell, D.J., 2003a. Axon terminals possessing the alpha 2c-adrenergic receptor in the rat dorsal horn are predominantly excitatory. Brain Research 965, 269–273.

Olave, M.J., Maxwell, D.J., 2003b. Neurokinin-1 projection cells in the rat dorsal horn receive synaptic contacts from axons that possess alpha2C-adrenergic receptors. Journal of Neuroscience 23, 6837–6846.

Olave, M.J., Maxwell, D.J., 2004. Axon terminals possessing alpha2C-adrenergic receptors densely innervate neurons in the rat lateral spinal nucleus which respond to noxious stimulation. Neuroscience 126, 391–403.

Ossipov, M.H., Harris, S., Lloyd, P., Messineo, E., Lin, B.S., Bagley, J., 1990. Antinociceptive interaction between opioids and medetomidine: systemic additivity and spinal synergy. Anesthesiology 73, 1227–1235.

Pan, Y.Z., Li, D.P., Pan, H.L., 2002. Inhibition of glutamatergic synaptic input to spinal lamina II(o) neurons by presynaptic alpha(2)-adrenergic receptors. Journal of Neurophysiology 87, 1938–1947.

Pertovaara, A., 2006. Noradrenergic pain modulation. Progress in Neurobiology 80, 53–83.

Przewlocka, B., Lason, W., Dziedzicka, M., 1990. Modulation of prodynorphin peptides release from the rat spinal cord in vitro. Neuropeptides 16, 201–206.

Rajaofetra, N., Ridet, J.L., Poulat, P., Marlier, L., Sandillon, F., Geffard, M., Privat, A., 1992. Immunocytochemical mapping of noradrenergic projections to the rat spinal cord with an antiserum against noradrenaline. Journal of Neurocytology 21, 481–494.

Russell, R.D., Leslie, J.B., Su, Y.F., Watkins, W.D., Chang, K.J., 1987. Continuous intrathecal opioid analgesia: tolerance and cross-tolerance of mu and delta spinal opioid receptors. Journal of Pharmacology and Experimental Therapeutics 240, 150–158.

Sallinen, J., Hoglund, I., Engstrom, M., Lehtimaki, J., Virtanen, R., Sirvio, J., Wurster, S., Savola, J.M., Haapalinna, A., 2007. Pharmacological characterization and CNS effects of a novel highly selective alpha2C-adrenoceptor antagonist JP-1302. British Journal of Pharmacology 150, 391–402.

Satoh, E., Nakazato, Y., 1991. Effects of monensin and veratridine on acetylcholine release and cytosolic free Ca^{2+} levels in cerebrocortical synaptosomes of rats. Journal of Neurochemistry 57, 1270–1275.

Shi, T.J.S., Winzer-Serhan, U., Leslie, F., Hokfelt, T., 1999. Distribution of alpha(2)-adrenoceptor mRNAs in the rat lumbar spinal cord in normal and axotomized rats. Neuroreport 10, 2835–2839.

Sinchak, K., Micevych, P.E., 2001. Progesterone blockade of estrogen activation of mu-opioid receptors regulates reproductive behavior. Journal of Neuroscience 21, 5723–5729.

Song, B., Chen, W., Marvizon, J.C., 2007. Inhibition of opioid release in the rat spinal cord by serotonin 5-HT(1A) receptors. Brain Research 1158, 57–62.

Song, B., Marvizon, J.C., 2003a. Peptidases prevent μ-opioid receptor internalization in dorsal horn neurons by endogenously released opioids. Journal of Neuroscience 23, 1847–1858.

Song, B., Marvizon, J.C.G., 2003b. Dorsal horn neurons firing at high frequency, but not primary afferents, release opioid peptides that produce μ-opioid receptor internalization in the rat spinal cord. Journal of Neuroscience 23, 9171–9184.

Song, B., Marvizon, J.C.G., 2005. NMDA receptors and large conductance calcium-sensitive potassium channels inhibit the release of opioid peptides that induce μ-opioid receptor internalization in the rat spinal cord. Neuroscience 136, 549–562.

Spike, R.C., Puskar, Z., Sakamoto, H., Stewart, W., Watt, C., Todd, A.J., 2002. MOR-1-immunoreactive neurons in the dorsal horn of the rat spinal cord: evidence for nonsynaptic innervation by substance P-containing primary afferents and for selective activation by noxious thermal stimuli. European Journal of Neuroscience 15, 1306–1316.

Stone, L.S., Broberger, C., Vulchanova, L., Wilcox, G.L., Hokfelt, T., Riedl, M.S., Elde, R., 1998. Differential distribution of alpha(2A) and alpha(2C) adrenergic receptor immunoreactivity in the rat spinal cord. Journal of Neuroscience 18, 5928–5937.

Stone, L.S., MacMillan, L.B., Kitto, K.F., Limbird, L.E., Wilcox, G.L., 1997. The alpha2a adrenergic receptor subtype mediates spinal analgesia evoked by alpha2 agonists and is necessary for spinal adrenergic-opioid synergy. Journal of Neuroscience 17, 7157–7165.

Summers, R.J., McMartin, L.R., 1993. Adrenoceptors and their second messenger systems. Journal of Neurochemistry 60, 10–23.

Takano, M., Takano, Y., Yaksh, T.L., 1993. Release of calcitonin gene-related peptide (CGRP), substance P (SP), and vasoactive intestinal polypeptide (VIP) from rat spinal cord: modulation by alpha 2 agonists. Peptides 14, 371–378.

Takemori, A.E., Portoghese, P.S., 1993. Enkephalin antinociception in mice is mediated by delta 1- and delta 2-opioid receptors in the brain and spinal cord, respectively. European Journal of Pharmacology 242, 145–150.

Trafton, J.A., Abbadie, C., Marek, K., Basbaum, A.I., 2000. Postsynaptic signaling via the mu-opioid receptor: responses of dorsal horn neurons to exogenous opioids and noxious stimulation. Journal of Neuroscience 20, 8578–8584.

Tricklebank, M.D., 2007. JP-1302: a new tool to shed light on the roles of alpha2C-adrenoceptors in brain. British Journal of Pharmacology 150, 381–382.

Uhlen, S., Xia, Y., Chhajlani, V., Felder, C.C., Wikberg, J.E., 1992. [3H]-MK 912 binding delineates two alpha 2-adrenoceptor subtypes in rat CNS one of which is identical with the cloned pA2d alpha 2-adrenoceptor. British Journal of Pharmacology 106, 986–995.

Uzumaki, U., Govoni, S., Faccini, E., Pasinetti, G., Missale, C., Trabucchi, M., 1984. Neuropeptidergic inhibitory regulation of Met-enkephalin immunoreactive material release from rat spinal cord in vitro. Peptides 5, 849–852.

Virtanen, R., Savola, J.M., Saano, V., Nyman, L., 1988. Characterization of the selectivity, specificity and potency of medetomidine as an alpha 2-adrenoceptor agonist. European Journal of Pharmacology 150, 9–14.

Wei, Z.Y., Karim, F., Roerig, S.C., 1996. Spinal morphine/clonidine antinociceptive synergism: involvement of G proteins and N-type voltage-dependent calcium channels. Journal of Pharmacology and Experimental Therapeutics 278, 1392–1407.

Yaksh, T.L., 1985. Pharmacology of spinal adrenergic systems which modulate spinal nociceptive processing. Pharmacology Biochemistry and Behavior 22, 845–858.

Zoli, M., Agnati, L.F., 1996. Wiring and volume transmission in the central nervous system: the concept of closed and open synapses. Progress in Neurobiology 49, 363–380.

Zorman, G., Belcher, G., Adams, J.E., Fields, H.L., 1982. Lumbar intrathecal naloxone blocks analgesia produced by microstimulation of the ventromedial medulla in the rat. Brain Research 236, 77–84.